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REMARKS

Claims 3-5 and 11-14 are pending in the subject application. The Examiner indicated in the January 30, 2006 Office Action that claims 3, 5 and 11-14 are in condition for allowance. No claim has been added, canceled, or amended herein. Accordingly, claims 3-5 and 11-14 are still pending and under examination.

**Rejection under 35 U.S.C. §112, First Paragraph and April 20, 2006
Examiner's Interview**

The Examiner rejected claim 4 under 35 U.S.C. §112, first paragraph, because the specification allegedly does not enable a method for preventing exaggerated restenosis in a diabetic human subject by administering to said subject any sRAGE polypeptide other than murine sRAGE *in vivo*.

Specifically, the Examiner states, in relevant part, that the sRAGE-mediated reduction of smooth muscle proliferation observed in the Fatty Zucker rat model used in the experiments underlying the subject invention is not predictive of similar results in a diabetic human subject.

This rejection was further discussed in the April 20, 2006 Examiner's interview. Applicants wish to thank the Examiner for his time and consideration during the telephonic interview with Alan J. Morrison, applicants' undersigned attorney.

During the interview, the Examiner indicated that submission of results of experiments involving the effect of sRAGE administration

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on smooth muscle proliferation in "large" animals (i.e., dog, pig or primate) would be predictive of results in a diabetic subject, and therefore, would be useful in overcoming the outstanding rejection.

In response, applicants respectfully traverse.

Applicants maintain that the results observed in the Fatty Zucker rats would be reasonably expected to be observed in human diabetic subjects. In support of this position, applicants attach hereto Touchard, et al. (Preclinical Restenosis Models: Challenges and Successes, Toxicology Pathology, 34:11-18 (2006)) (**Exhibit A**) and Park, et al. (Neointimal Hyperplasia After Arterial Injury in Increased in a Rat Model of Non-Insulin-Dependent Diabetes Mellitus) (**Exhibit B**).

Page 16 of Touchard, et al. states that the "fundamental parameter [in an ideal model]...is the amount of neointimal hyperplasia. Models with more neointima are typically the best models." Page 817 of Park, et al. states that the "Zucker rat model, which is a prototype for type II diabetes...exhibited a marked tendency to form neointima after balloon injury compared with the lean controls." Applicants maintain that given the neointimal hyperplasia seen in the Zucker rats, these animals constitute an ideal animal model for exaggerated restenosis in a diabetic human.

Furthermore, applicants assert that results obtained from "large" animal experiments, as requested by the Examiner, would not necessarily be more predictive than the results obtained from Fatty Zucker rats.

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For example, regarding a canine model for restenosis, page 12 of Touchard, et al. states that "dogs have high fibrinolytic activity markedly different from the human coagulation system. In addition, the canine vessel wall produces only a thin neointima when compared with other animal models. *These considerations make the dog a poor model for restenosis*" [emphasis added].

Regarding a nonhuman primate model for restenosis, page 12 of Touchard, et al. also states that the "temporal sequence of proliferative response and thrombotic activity in coronary arteries [of nonhuman primates] is not well related to human restenosis and other animals." Accordingly, the nonhuman primate model for restenosis would not be as predictive as the Fatty Zucker rat.

Regarding the porcine model for restenosis, applicants maintain that diabetic pigs do not exhibit the fundamental parameter discussed in Touchard, et al. required in an accurate diabetic animal restenosis model, i.e., a high level of neointimal formation. In support of this position, applicants attach hereto Carter, et al. (The effects of uncontrolled hyperglycemia on thrombosis and formation of neointima after coronary stent placement in a novel diabetic porcine model of restenosis, Coronary Artery Disease, Sept. 11(6):473-479 (2000)) (**Exhibit C**). Page 478 of Carter, et al. states that "the 28-day neointimal responses to oversized stenting in the surviving diabetic and [in] age-matched nondiabetic control swine were similar" (See also Figures 2 and 3 of Carter, et al.). Furthermore, page 478 of Carter states that the diabetic pigs "do not have hyperinsulinemia, which is typical for a patient with type II diabetes. Hyperinsulinemia...might contribute significantly to the exaggerated neointimal response to

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coronary stenting in diabetic patients." As such, applicants maintain that diabetic pigs do not provide as accurate an animal model for restenosis as does the Fatty Zucker rat.

In sum, applicants maintain that results obtained from experiments from these "large" animals (i.e. dog, pig or nonhuman primate) would not be any more predictive, and more likely would be less predictive, than the results obtained from the Fatty Zucker rat experiments. Applicants maintain that the Fatty Zucker rat is a predictive animal model for human restenosis and based on the rat experiments in the specification, one skilled in the art would reasonably expect to observe a prophylactic result in human diabetic patients.

In view of the above, applicants maintain that claim 4 satisfies the requirements of 35 U.S.C. §112, first paragraph.

Summary

For the reasons set forth hereinabove, applicants maintain that claim 4 is in condition for allowance, and respectfully request allowance.

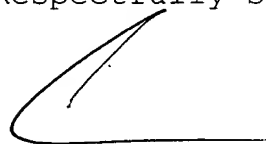
If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee, other than the \$510.00 fee for a three-month extension of time, is deemed necessary in connection with the filing of this

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Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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Preclinical Restenosis Models: Challenges and Successes

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ABSTRACT

Coronary artery disease remains a major problem for Western societies. The advent of percutaneous interventions, including stents has brought clinical care to a new level of efficacy, yet problems remain. Restenosis following stenting in human coronary arteries appears at last to be yielding to therapeutic strategies, especially drug eluting stents. Because therapeutic percutaneous coronary intervention is widely dominated by the intracoronary stent, restenosis therapies must include the stented coronary artery. Animal models and in particular the porcine coronary model seem to represent the human coronary artery reaction to stenting. It mimics several clinical conditions including thrombosis and neointimal formation. A key question in the era of intravascular technologies is how well this and other models can predict clinical events. This paper discusses the models and their application.

Keywords. Neointima; restenosis; stent; vascular injury

INTRODUCTION

Research in human coronary atherosclerosis is limited by an inability to control experiments and by the slow temporal lesion development. Fortunately, animal arterial injury models appear to yield comparable results to clinical trials, and can teach about the arterial response to injury. These models have become indispensable for understanding the interaction of the coronary artery with medical devices, and toward understanding neointimal genesis. They can likely function to test safety and efficacy of new devices. In these models the pathophysiologic aspects of disease can be simulated, variables can be controlled, and statistical data accrued in short time periods.

Many animal models have been used for restenosis studies. This variety comes because an ideal animal model does not exist. Each animal model has advantages and disadvantages. This chapter discusses the principal animal models described for restenosis studies, their characteristics, advantages and disadvantages compared with humans, and the considerations necessary for proximity to an ideal animal model and study design.

Preclinical Restenosis Models

Common animal models used for restenosis studies include rodents (rats, mice, rabbits), pigs, dogs, or primates. Frequent injury methods used in these models include mechanical injury (overstretch artery with noncompliant angioplasty balloons inflated to high pressures, very compliant, low-pressure balloons for denudation injury (Rogers et al., 1993), wire loops (Reidy and Schwartz, 1981; Walker et al., 1983; Lindner et al., 1991; Lindner and Collins, 1996; Lindner and Reidy, 1996) or directional atherectomy) or injury induced by agents such as chemical-diet-, electrical injury (Carmeliet et al., 1997), heat (Douek et al., 1992), air desiccation, Fishman et al., 1975; Gellman et al., 1991; Sarembock et al., 1996 irradiation (Fajardo and Berthrong, 1988) or inducing severe inflammation with copper stents by

foreign body implant (Ide et al., 1994; Schwartz et al., 1992a; Schwartz, 1994; Staab et al., 1997).

To enhance lesion formation or to reproduce conditions that predispose to the need for human arterial angioplasty (such as atheroma presence), before or after the "principal" injury, other authors have developed complementary injurious methods. Animals can be placed on a high-fat, high-cholesterol diet (chemical injury) or undergo other nondietary injury modes, alone or in tandem with the cholesterol diet. These create double or triple injury as models. It is unclear if such complementary injuries may positively or negatively affect final results of a study.

RODENTS

Rat Carotid Artery Model

Extensive studies on the response to vascular injury were performed in the rat carotid artery model years before angioplasty became known. These studies, based on denudation injury with a very compliant, low-pressure balloon, identified the intimal layer as a key site in the proliferative response. In this model, both carotid arteries are typically used in the same animal (Figure 1). The rat carotid artery is injured either by air desiccation (Gellman et al., 1991; Sarembock et al., 1996) or by balloon endothelial denudation (Au et al., 1992; Clowes et al., 1991; Golden et al., 1990). A 2F Fogarty balloon is advanced through an incision in the external carotid artery to the common carotid artery. The balloon is inflated and drawn through the artery (while inflated) for multiple passes, generally 3 or more times. The balloon is deflated and removed, and the external carotid artery is ligated.

The Hypercholesterolemic Rabbit Iliac Model

The rabbit atherosclerotic iliac restenosis model has been used commonly. Although the lesions of this model differ from human lesions, it provides valuable insights for understanding the mechanism of repair after injury to an abnormal artery and for testing restenosis therapies (Baumbach et al., 1997; Coats et al., 1996; Hansen et al., 1988; Jenkins et al., 1989; Kalinowski et al., 2001; Kanamasa et al., 2001; Nagae et al., 2001; Welt et al., 2000; Zou et al., 2000). Rabbit models are typically single, double- or even triple-injury models, and

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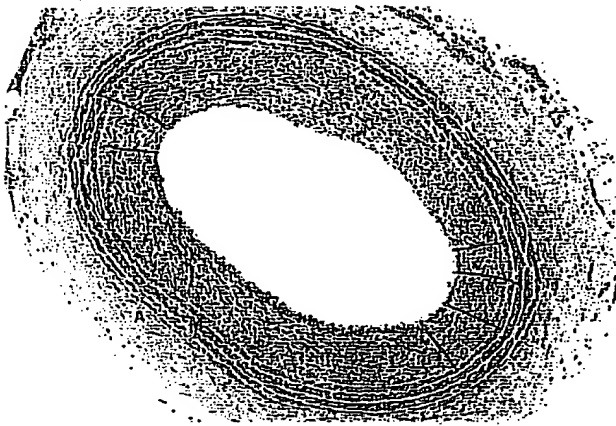


FIGURE 1.—Rat carotid artery. The three basic layers of the wall can be seen: the tunica intima I, the tunica media M, and the tunica adventitia A. The inner layer is the intima, the outer layer (concentric white bands) is the adventitia. In between is the tunica media. Note the pink staining, corresponding to the elastic laminae (asterisk) in the tunica media, which is typical for the elastic arteries such as the carotid artery. H&E staining.

include biochemical injury with hypercholesterolemic diets is followed by mechanical injury into both femoral arteries with a balloon catheter or sometimes air desiccation. Four to 6 weeks after injury, lesions are evaluated for stenoses. If a significant lesion is found, an angioplasty (second mechanical injury) is performed under fluoroscopic guidance.

The Dog: Minimal Response to Injury

Dogs have been explored as an experimental model for restenosis mainly because of their size, cost, and ready availability. However, dogs have high fibrinolytic activity (Mason and Read, 1971), markedly different from the human coagulation system (Kirschstein et al., 1989). In addition, the canine vessel wall produces only a thin neointima when compared with other animal models (Schwartz et al., 1994) (Figure 2). These considerations make the dog a poor model for restenosis.

Porcine Coronary Injury Model (Figure 3)

The porcine heart and its coronary artery system have a size and anatomical structure very similar to that of humans (Ali et al., 1996; Schwartz, 1998; Schwartz et al., 1993).

The carotid arteries are typically used for arterial access in this model, although the femoral arteries may also be used without difficulty. Standard guide catheters and curves for human coronary angioplasty are used in both techniques for engagement of the left main or right coronary arteries, which is a great advantage of these models.

Mechanical injury by oversizing the artery and endothelial denudation alone has proven successful, but oversizing is a stronger stimulus for smooth muscle cell proliferation than endothelial denudation alone. Oversizing the coronary artery can be achieved using a coronary angioplasty balloon (Heras et al., 1989; Schwartz et al., 1990) or oversized stent implantation (McKenna et al., 1998). This model produces a neointimal response virtually identical to human restenotic neointima in terms of cell size, cell density, and histopatho-

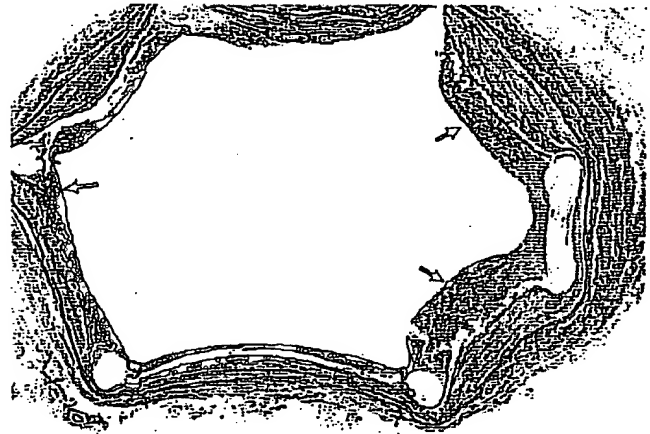


FIGURE 2.—Dog coronary artery after severe mechanical injury. Despite deep vessel wall injury partially extending to the external elastic lamina, the amount of neointima is minimal and clearly nonobstructive (arrows). Elastic van Gieson stain.

logic appearance (Schwartz et al., 1990, 1992, 1993, 1994). Specimens from balloon-only injury typically show a single laceration of media, and specimens from oversized stent implantation show multiple injuries in the neighborhood of the stent wires, also very similar to human findings.

Nonhuman Primates

Nonhuman primates bear phylogenetic resemblance to humans, a potentially singular advantage. The temporal sequence of proliferative response and thrombotic activity in coronary arteries is not well related to human restenosis and other animals. Their limited availability, legal restrictions, ethical concerns, and high cost, make this animal model impractical. Few studies using primate arteries to balloon catheter injury have thus been reported (Geary et al., 1995, 1996, 1998; Hanson et al., 1991; Mondy et al., 1997).

Species-Specific Arterial Response to Injury?

Not surprisingly, species have individualized molecular and cellular arterial healing mechanisms, and so vascular lesions following injury differ immensely across these species. Rats, mice and porcine carotid injury models almost never form hemodynamically significant stenoses. In hypercholesterolemic rabbits and porcine coronary arteries, macroscopic and hemodynamically significant stenoses may develop, but it does not occur systematically.

The arterial response to injury typically occurs in 6 phases: (1) arterial damage (endothelial denudation, internal elastic lamina fracture, media injury, adventitial injury), (2) platelet aggregation and thrombus formation, (3) elastic recoil, (4) inflammation, (5) smooth muscle cell migration, proliferation and extracellular matrix production (Clowes et al., 1989), the principal responsible of the neointimal thickening (Casscells, Clowes, and Schwartz, 1990; Fingerle et al., 1989; Hanke et al., 1990), and (6) arterial remodeling.

Each of these factors contributes to restenosis following angioplasty alone (Faxon and Currier, 1995; Landzberg et al., 1997). However, following stent placement, endothelial

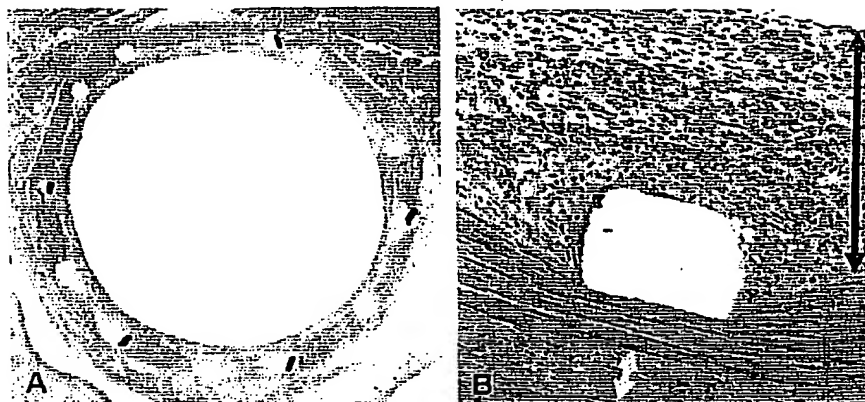


FIGURE 3.—Pig coronary artery, 28 days after mechanical injury (stent oversizing). Micrograph (A) illustrates mechanical injury 28 days after stent oversizing. Micrograph (B) illustrates the neointimal response (blue arrow) in relationship to the media (green arrow) and adventitia (yellow arrow). H&E.

damage, thrombosis, inflammation and intimal hyperplasia appears to be the predominant pathology (Braun-Dullaeus et al., 1998; Gershlick and Baron, 1998). No single model appears to have all component processes identical to humans. Species-specific differences in arterial healing must be considered in study design and interpretation. Failure to account for these can cause confusion, potential data misinterpretation, or errors. Restenosis pathophysiology is described elsewhere in this book. The purpose of the present section is to highlight pathophysiologic differences between species.

Arterial Damage and Injury Score

All types of arterial injury begin with endothelial denudation, and progress to deeper injury. The degree and

susceptibility to deeper injury exhibits species specificity. In the rat carotid model, injury typically shows endothelial denudation, remaining intact other arterial structures such as the internal elastic lamina, media, and external elastic lamina (Figure 1) (Lindner et al., 1989). This mild injury contrasts to deeper arterial injury usually observed in the rabbit iliac and porcine coronary arteries (Figures 3 and 4), where internal elastic lamina and medial dissection is similar to the endothelial and medial damage following human percutaneous coronary intervention.

The type of injury may also induce different arterial injury degree even in the same animal. For example, arterial injury in rats is different, using a wire loop were only endothelial denudation is seen. This is compared with electric injury where

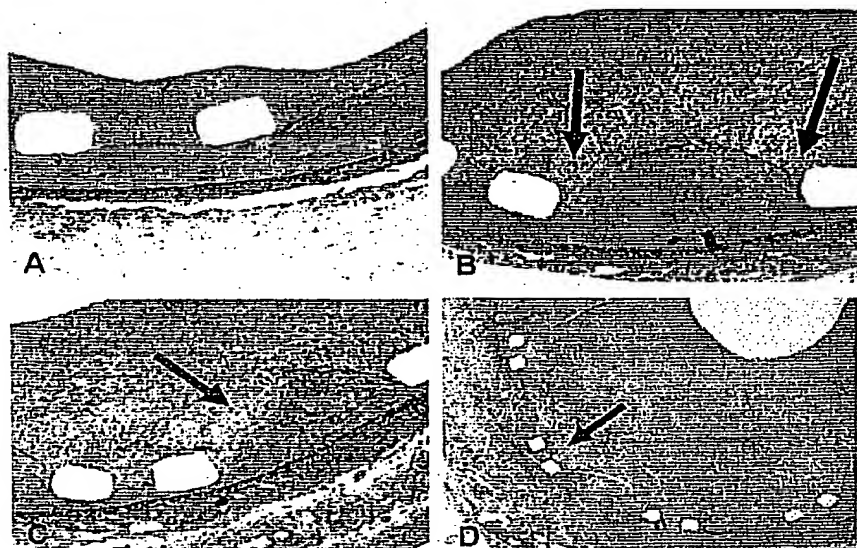


FIGURE 4.—Injury scoring for the porcine coronary artery model. Micrograph (A) illustrates an injury score of zero characterized by superficial vessel wall damage with intact external lamina. Micrograph (B) illustrates an injury score of 1 characterized by fracturing of the internal elastic lamina by stent wires (arrows) with intact media. Micrograph (C) illustrates an injury score of 2 characterized by media laceration (arrow) with intact media. Micrograph (D) illustrates an injury score of 3.

wide necrosis zones from intima to adventitia can be seen. This different injury could stimulate different arterial healing phases in the same animal models.

Severity of mechanical injury across animal models might account for variability in neointimal hyperplasia. A porcine coronary injury score (Figures 4 and 5) based on the integrity of the structural components of the vessel wall has resulted from such observations. This progressively relates superficial vessel wall damage (injury score of zero) to newly formed neointima that is very thin, as occurs with appropriately sized stenting. Stenoses develop progressively only when stent wires fracture the internal elastic lamina (score 1), or lacerate the media (score 2) or the external elastic lamina (score 3). It is unknown whether the elastin membranes influence the biomolecular aspects of neointima formation or if it can be regarded only as a marker for injury severity. There is evidence that the internal elastic membrane may function as a barrier for the diffusion of macromolecules from the lumen and as a base for the attachment of endothelial cells (Sims, 1989).

The injury score can be used to compare studies and quantitate the response to potential therapies (Schwartz et al., 1994, 1996b) (Huber et al., 1993). The peripheral arteries have been used similarly in examining the arterial response to injury (Yang et al., 1996; Schwartz et al., 1996a; Kullo et al., 1997).

Thrombus Formation—The Importance of the Thrombotic and Fibrinolytic Response

After arterial injury, the clotting and fibrinolytic mechanisms are activated. This response to vessel wall injury is substantially different across species (Kirschstein et al., 1989; Mason and Read, 1971; Schwartz et al., 1992b, 1993), and different amounts of mural thrombus can be seen depending on the animal model used. In the rat carotid and canine models, significant fibrin-rich thrombus is rarely if ever found. Conversely, in the rabbit iliac and porcine model, macroscopic thrombus does occur and has been characterized in several reports. Primates have comparable fibrinolytic or hemostatic systems to humans. For example, baboons are prone to acute stent thrombosis within the first 3 days after the procedure, which is significantly different than the acute stent occlusion in pig arteries, which usually occurs within 6 hours (Mason and Read, 1971; Schwartz 1998; Schwartz and Holmes, 1994).

Mural thrombus provides a scaffold for medial smooth muscle cell colonization. According to this concept, the amount of mural thrombus might govern the total neointimal burden. This could explain, among other factors, why rat carotid and dog coronary arteries may not generate substantial neointimal volume (and macroscopic stenoses) in distinction to the rabbit and porcine models. Against this theory, is that numerous anticoagulation trials have failed to impact restenosis, including warfarin, heparin, and direct thrombin inhibitors. However, whether these regimes eliminate local thrombus formation is unknown.

Differences in mural thrombus formation after angioplasty must be present in the design and interpretation of antithrombotic agents in various models. Animal models with greater tendency to thrombus formation (pigs, for example) can be

more sensitive to antithrombotic agents than humans. It is thus possible that antithrombotic agents are effective for pigs but not for humans.

Several examples exist in the literature, such as prostacyclin (Colombo et al., 2003), aspirin (Clopath, 1980) and hirudin (Abendschein et al., 1996; Buchwald et al., 1996; Gallo et al., 1998) or low molecular weight heparin (Buchwald et al., 1996), where studies performed in pigs, show some efficacy of hemostatic interventions in the reduction of restenosis that failed in humans (Bittl et al., 1995; Serruys et al., 1995; Johnson et al., 1999). However, as discussed later, different endpoints in the animal studies may cause differences from human studies. Since mural thrombus may provide a scaffold for medial smooth muscle cell colonization, the tendency to thrombus formation may be an explanation why some antiproliferative therapies demonstrate significant inhibition of neointimal hyperplasia in some animal models that do not translate to clinical trials. In summary, more or less thrombus formation must be present when choosing an animal model for antithrombotic therapy testing and in the extrapolation of the results in humans.

Inflammation

Few studies document the role of inflammation in restenosis, although it is key. In addition, inflammation resolution is also important since it may produce a scar fibrosis, and resultant negative remodeling.

In the rat carotid model of injury there is remarkably little inflammatory response to injury. Hypercholesterolemic rabbits, porcine and nonprimate models show robust inflammatory reactions to injury (Figure 5), with early mononuclear cell infiltration from the lumen into the thrombus (Schwartz et al., 1992b; Miyauchi et al., 1998). In the porcine model inflammation is positively related to neointimal thickness (Kornowski et al., 1998). Human studies of stented arteries also show acute inflammation early after implantation, especially when stenting is associated with medial injury or lipid core penetration (Komatsu et al., 1998; Farb et al., 1999; Grewe et al., 2000). Macrophage infiltration in atherectomy tissue and the activation status of blood monocytes correlate with an increased rate of restenosis (Moreno et al., 1994; Pietersma et al., 1995).

Smooth Muscle Cell Proliferation and Migration

Although rat and mouse studies initiated the concept of proliferation, such proliferation and migration from media to intima, is considered a prominent feature in all animal models. Despite the observation that SMC neointima formation causes in-stent restenosis, the role of cell proliferation within the neointima remains controversial.

It is uncertain if there is a species-specific cell proliferation and migration. Cell proliferation and migration in rats mice and pigs begins early after denudation (1 or 2 days) and proceeds for the following 14 to 30 days (peaking in 2–3 weeks); (Fingerle et al., 1989; Schwartz, 1992b; Zempo et al., 1996). The Rabbit iliac model shows proliferation over the same period with peak at 8 days (Stadius et al., 1994). In non-human primates, proliferation is increased at 4 and 7 days but later declines to control rates (Geary et al., 1996). Regardless of the fact that animal models show a hyperplastic response to injury, kinetics of cell proliferation in human vessels does

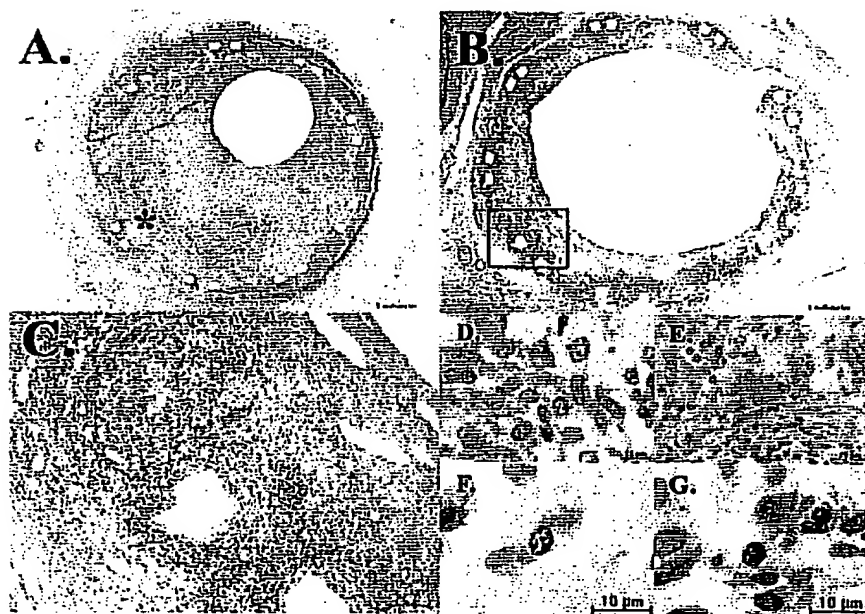


FIGURE 5.—Example of chronic vascular inflammation. (A) Granulomatous reactions in one artery around the penetrating struts (red asterisk). (B) Generalized granulomatous reaction in a stent venous section. (C) Magnified image ($\times 100$) of red square of B shows typical granulomatous reaction (see text). (D) Macrophages of Figure C ($\times 400$). (E) Giant cell of figure C (yellow asterisk) ($\times 200$). (F) Plasma cells of Figure C ($\times 800$). (G) Eosinophils of Figure C ($\times 800$). A and B, $\times 20$ Movat staining; C to G, H&E staining.

not appear well defined. Human lesions show a comparative hypocellular response with abundant matrix.

Elastic Recoil and Remodeling

Acute elastic recoil immediately following balloon deflation and late vascular constriction (negative remodeling); (Lafont et al., 1995; Mintz et al., 1996; Bauters and Isner, 1997; Schwartz et al., 1998) occur with PTCA alone, and are important aspects of restenosis pathophysiology in the pre-stent era. Coronary stents acting as a mechanical scaffold within the vessel, eliminating elastic recoil and vessel contracture (Kay et al., 2000; Shah et al., 2002). Hypercholesterolemic rabbit (Kakuta et al., 1998; Kalef-Ezra et al., 2002), porcine coronary artery (Waksman et al., 1997) and nonhuman primates (Coats et al., 1997) exhibit remodeling behavior in much the same fashion as man (Schwartz et al., 1998). Mouse arteries tend to enlarge after angioplasty and appear naturally prone to positive remodeling (Carmeliet et al., 1997; de Smet et al., 1998).

Regardless that remodeling is lost in the stent era, animal models prone to positive or negative remodeling must be a consideration today since positive vascular remodeling occurs after bare-metal stent implantation (Shah et al., 2002), after catheter-based radiation followed by conventional stent implantation (Kay et al., 2000) and but not demonstrated, after drug-eluting stent implantation.

The ideal experimental model to assess restenosis treatment would be one that reliably predicts the risks and outcome of human clinical trials. Although such an ideal model does not yet exist, experimental studies are ongoing. Many pharmacologic agents, such as antiplatelet, anticoagulants, ACE inhibitors and antiproliferative drugs have been tested

successfully in animal models failed in human clinical trials. The marked disparity of results between animal model research and clinical trials has led to skepticism about the validity of animal models in restenosis research.

The failure of animal studies to predict efficacy in preventing human restenosis is potentially attributable to two general factors. Species differences may in part be responsible, a factor not easily modified except with transgenic animals. Second there are several modifiable factors, not taken into account, which are able to approach the currently models to the ideal animal model.

Unknowns in Models

The importance of several biologic factors variability between species in restenosis or in the positive or negative predictive values are unknown. The impact of concomitant atherosclerosis in animal models is not well defined. Restenosis can be studied on either normal or previously injured arteries. The most commonly employed technique is the arterial injury over a normal coronary artery. The normal coronary artery of a young rat, rabbit, or pig differs distinctly from the atherosclerotic coronary artery of an older patient. Arteries of these animal models, even those of with hyperlipidemic diets (developing during a few weeks instead of decades as in humans), do not show densely fibrous and acellular plaques with ulceration, calcification, thrombosis, or hemorrhage into the vessel wall, all features of human restenosis. The impact of this atherosclerotic environment on restenosis and whether the use of models that produce atherosclerosis will have advantages over nonatherosclerotic models is also unknown.

Another consideration is the impact of protective molecules against atherosclerosis in restenosis models such

cholesteryl ester transferase or the high levels of HDL and low levels of LDL in mice. Cholesteryl ester transferase is present in humans, swine, and rabbits and is deficient in dogs and rodents. This enzyme explains in part the difficulty in inducing atheroma lesions in these latter animals (Tall, 1986; Narayanaswamy et al., 2000).

CONCLUSIONS—THE IDEAL MODEL

Many catastrophic articles in the last decade have been written about the failure of animal models in predicting results in human trials. These have generated distrust in the current animal models, but little true data exist about the true place for these models and their relation to humans. There is no doubt that animal models have significantly advanced our understanding of the mechanisms of restenosis formation and have served to improve the therapeutic options.

For the moment, a single ideal global model does not exist, but promising research is ongoing in this area. At present, compromises in choosing an animal model are inevitable. A detailed species understanding for limitations and strength features must be considered for the specific purpose of the study (for example thrombosis vs. migration), for the results interpretation and for human extrapolation.

Taking into account all data commented in the present chapter, the "ideal model" should instead be considered of an "ideal study." A rational approach to the ideal study is important:

1. Arterial response to injury (pathogenesis) studies: Multiple animal models are valid. Since this model serves to create hypothesis and subsequently new treatment strategies, minimal arterial changes may be sufficient. New hypothesis and treatment strategies should be confirmed in animal models that demonstrate good predictive values, such as the coronary porcine model.
2. Safety studies: prior comments about artery type, devices, dosage and drug timing, animal pathophysiologic differences must be considered. More close alignment with human stenting is strongly recommended. For this purpose, not all animal models would be valid.
3. Efficacy studies: The fundamental parameter besides safety studies is the amount of neointimal hyperplasia. Models with more neointima are typically the best models. It is of paramount importance to assess histologic information along the same human endpoints such as angiographic restenosis or IVUS. All results should be regularly reported and carefully evaluated.

To date, considering all models, the porcine overstretch coronary artery appears closest to humans for global use since its coronary anatomy, physiology and pathophysiology. In addition, it permits using of the same devices used in humans and produces the thickest neointima in response to injury.

Animal models will continue to provide more complete understanding of restenosis and to find the improved therapies for human restenosis. However, efforts for developing improved animal models must continue. New animal models which are now under investigation may provide additional insight into new and important aspects of animal models that could predict the success of therapeutic interventions in animals and ultimately in humans.

ACKNOWLEDGMENTS

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Neointimal Hyperplasia After Arterial Injury Is Increased in a Rat Model of Non-Insulin-Dependent Diabetes Mellitus

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Neointimal Hyperplasia After Arterial Injury Is Increased in a Rat Model of Non-Insulin-Dependent Diabetes Mellitus

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Background—The key biological determinants that promote restenosis in the setting of diabetes have not been elucidated. There is no accepted animal model to study restenosis in diabetes.

Methods and Results—We evaluated 2 models of diabetes mellitus: (1) streptozotocin (STZ)-treated Sprague-Dawley rats (type I diabetes) versus regular Sprague-Dawley rats and (2) obese Zucker rats (type II diabetes) versus lean Zucker rats. Neointimal hyperplasia was assessed after carotid balloon injury at 21 days by computerized morphometry. There was no difference in neointimal area in the STZ-treated rats compared with controls, irrespective of insulin administration or dose of STZ. Neointimal area was increased >2-fold in obese Zucker rats compared with lean Zucker rats (0.21 ± 0.06 versus 0.08 ± 0.03 mm², $P < 0.01$). The neointimal area was markedly increased in the obese Zucker rats 7 days after injury (0.058 ± 0.024 versus 0.033 ± 0.009 mm², $P < 0.05$) and persisted through 21 days. In both obese and lean Zucker rats, cell proliferation peaked in the media at 3 days (118.66 ± 84.28 versus 27.50 ± 12.75 bromodeoxyuridine-labeled cells per cross section). In the intima, cell proliferation markedly increased beginning at day 3 and persisted through day 14 in the obese and lean Zucker rats (202.27 ± 98.86 versus 35.71 ± 20.54 bromodeoxyuridine-labeled cells at 7 days).

Conclusions—The type II diabetic rat model, typifying insulin resistance, is associated with a propensity for neointima. The obese Zucker rat model may be an ideal diabetic model to further characterize the diabetic vascular response to injury. (*Circulation*. 2001;104:815-819.)

Key Words: diabetes mellitus ■ restenosis ■ insulin ■ angioplasty

Diabetic patients have an increased incidence of acute complications, late myocardial infarction, restenosis, and mortality after percutaneous coronary intervention.¹⁻⁵ Intravascular ultrasound studies demonstrate that neointimal proliferation, after both balloon angioplasty and stenting, results in greater restenosis rates in diabetic patients. The biological determinants of restenosis among patients with diabetes mellitus, however, are unknown. Therefore, we developed type I and II diabetic animal models of arterial injury to investigate the pathobiology of restenosis in diabetic patients. We hypothesized that both diabetic models would have enhanced neointimal proliferation compared with control animals. We also examined potential biological contributors to coronary restenosis in diabetes, such as insulin resistance, exogenous insulin administration, and glucose, cholesterol, and triglyceride levels. We characterized the mechanisms of the pathogenesis of restenosis in diabetes with regard to smooth muscle cell proliferation.

Methods

Type I (Insulin-Dependent) Diabetic Animals

To make a type I diabetic rat model, we injected streptozotocin (STZ) into Sprague-Dawley rats, followed by arterial injury 2 weeks

later. Male Sprague-Dawley rats 11 to 14 weeks old were selected at random for intravenous injection of STZ in 50 mmol/L citrate buffer (pH 4.5) or citrate buffer alone (control group). Animals were weighed immediately before STZ injection. We used 2 different doses of STZ, a relatively low dose (40 mg/kg) and a high dose (60 mg/kg), to simulate 2 diabetic rat groups with various degrees of insulin deficiency.^{6,7} These 2 type I diabetic rat groups were each compared with separate control groups in separate experiments. STZ-injected rats were further divided into 2 groups according to whether or not they received insulin treatment. Rats in the insulin-treated diabetic cohorts were treated subcutaneously with 2 U NPH insulin for the low-dose-STZ group and 4 U NPH insulin for the high-dose group, beginning 1 week after injection of STZ. All animals were allowed free access to food and water. Animals were reweighed weekly. A blood glucose level of ≥ 250 mg/dL, documented 1 week after injection of STZ, was required for inclusion of the diabetic animal in the study. Animals were euthanized 3 weeks after arterial injury for assessment of neointimal hyperplasia.

Type II (Insulin-Resistant) Diabetic Animals

The obese Zucker rat, characterized by excessive body weight, insulin resistance, hyperinsulinemia, and mild hyperglycemia, is a well-established model of type II diabetes.⁸ We included obese Zucker rats 8 to 9 weeks old for a type II diabetic model and lean Zucker rats 11 to 14 weeks old as controls. Experiments were

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performed in 2 separate sessions. We first evaluated neointimal hyperplasia 3 weeks after arterial injury and then, in separate experiments, we assessed cell proliferation at 1, 3, 7, 14, and 21 days after injury.

Surgical Procedure and Tissue Preparation

Arterial injury was performed by balloon deendothelialization. After induction of anesthesia with an intraperitoneal injection of xylazine (4.6 mg/kg) and ketamine (70 mg/kg), a midline cervical incision was made to expose the left external carotid artery. With a 2F Fogarty balloon catheter (Baxter Healthcare Corp), carotid artery injury was performed according to a previously described technique.⁹ After anesthesia, animals were euthanized through a midline abdominal incision exposing the distal abdominal aorta. With an 18-gauge intravenous catheter introduced at the aortic bifurcation, the aorta was flushed with 50 mL of Ringer's lactate solution at 120 mm Hg, followed by *in vivo* fixation with 200 mL of 5% Histochoice (Amresco) infused over 5 minutes at 120 mm Hg. Histochoice is an acid aldehyde with 10% alcohol solution. Once the perfusion-fixation was started, the animals were killed with an overdose of thiopental sodium through the tail vein. After 5 minutes of perfusion-fixation, the entire left carotid arteries were harvested, including the aortic arch, innominate artery, and carotid bifurcation. The specimens were stored in 5% Histochoice until sectioning. The injured common carotid arteries were cut every 3 mm from the aortic arch to the bifurcation into the external and internal carotid arteries. Five segments were embedded in paraffin for sectioning, and duplicate slides were stained with hematoxylin-eosin and elastic van Gieson stain. Three different segments, with the maximal neointimal proliferation of the left common carotid artery, were selected for histological, morphometric, and immunohistochemical studies.

Histomorphometric Study

Morphometric analysis of the arterial segments was carried out by an observer blinded to the study group, and quantitative measurements were performed on the segment that exhibited the greatest area of neointima. With a computerized digital microscopic planimetry algorithm (NIH Image 1.56), cross-sectional areas of the lumen, intima, media, and vessel circumscribed by the external elastic lamina were measured. Intimal cell counting was performed with a previously validated method.¹⁰ Analyses were done on cross sections stained with hematoxylin-eosin under $\times 40$ microscopic magnification. Random areas (encompassing 20% to 40% of the total intimal cross-sectional area) within the intima were selected, and cell nuclei were enhanced and counted after dynamic color thresholding. The average cell nuclear count within these known areas was used to calculate the cell density (cells/mm²), which, when multiplied by the previously measured total intimal area (from elastic van Gieson-stained sections), was used to calculate the total intimal cell count.

BrdU Injection and Immunohistochemistry

To detect proliferative cells at each time point after vascular injury, we injected bromodeoxyuridine (BrdU) (Sigma Chemical Co) and performed immunohistochemistry¹¹ at each of the 5 time points after balloon injury. The BrdU antibody dilution was 1:100. BrdU was administered at 18 hours (30 mg/kg IP and 100 mg/kg SC) and 12 hours (30 mg/kg IP) before euthanasia. Immunohistochemistry was performed with a monoclonal antibody (Dako Co) to BrdU. BrdU-labeled cells were counted in media and intima at $\times 200$ magnification. In the intima, the BrdU-labeling index (the fraction of labeled nuclei times 100) was calculated to assess cell replication rate. The temporal and spatial presence of BrdU-labeled cells was observed in the media and intima to assess cell migration after carotid balloon injury.

Blood Chemistry Assay

Blood glucose levels were checked twice weekly for 2 weeks after injection of STZ and weekly thereafter. Blood glucose was measured with a standardized, portable glucometer in blood obtained from the tail vein. Serum was obtained from nonfasted animals at death and

frozen at -20°C until assay. Total cholesterol was measured by the cholesterol oxidase enzyme assay, triglycerides by the glycerol triphosphate oxidase enzyme assay, and insulin levels by radioimmunoassay with an antibody made specifically against rat insulin (Linco). The rat insulin antibody has 100% cross-reactivity with porcine insulin. Ketone bodies were checked by the nitroprusside reaction.

Statistical Analysis

All data were expressed as mean \pm SD. All statistical analyses were performed with Stat-View Version 4.5 (Abacus Concepts, Inc). The unpaired Student's *t* test and Fisher's exact test were used to compare parametric data and nonparametric data, respectively, between 2 groups. When comparisons of >2 groups were required, statistical significance was determined by ANOVA. A value of $P < 0.05$ was considered significant.

Results

Sixty-two obese Zucker rats, 60 lean Zucker rats, and 140 Sprague-Dawley rats were started on the experimental protocol. A total of 55 obese Zucker rats and 55 lean Zucker rats survived until the time of euthanasia. Twelve Sprague-Dawley rats were excluded for inadequate glucose levels after STZ injection, 28 died during surgery, and an additional 15 died in the follow-up period. A total of 85 Sprague-Dawley rats thus survived until euthanasia. The type I diabetic rats without exogenous insulin treatment exhibited marked weight loss, which was related to the dose of STZ (Table 1). The glucose, triglyceride, cholesterol, and insulin levels are depicted in Table 1.

Morphometry at 21 Days After Carotid Balloon Injury in Diabetic Rats

In the type I diabetic rats, irrespective of insulin treatment and dose of STZ, there were no significant differences in neointimal hyperplasia 21 days after carotid balloon injury compared with controls. There were also no differences in lumen, external elastic lamina, or medial areas (Table 2). In contrast, the neointimal area was increased >2 -fold in the type II diabetic obese Zucker rats compared with control lean Zucker rats (0.21 ± 0.06 versus 0.08 ± 0.03 mm², $P < 0.01$). There were no differences in luminal or medial areas between obese and lean Zucker rats, but the external elastic lamina area was larger in obese Zucker rats (Table 2).

Time Course of Neointimal Formation in Type II Diabetic Rats

Compared with lean Zucker rats, neointimal area was increased in type II diabetic obese Zucker rats beginning 7 days after injury (0.058 ± 0.024 versus 0.033 ± 0.009 mm², $P < 0.05$) (Figure 1). Differences in neointimal area became progressively greater in obese versus lean Zucker rats and continued to diverge through 21 days (0.20 ± 0.03 versus 0.10 ± 0.03 mm², $P < 0.01$).

Cell Proliferation

The number of proliferating BrdU-labeled cells in the media was highest in obese Zucker rats relative to lean Zucker rats at 3 days (118.66 ± 84.28 versus 27.50 ± 12.75 cells per cross section, $P < 0.05$) and remained significantly higher in obese Zucker rats at 7 days (29.36 ± 26.44 versus 1.14 ± 1.21 cells per cross section, $P < 0.05$) (Figure 2). In the intima, the

TABLE 1. Animal Characteristics and Laboratory Findings

	OZ (n=12)	LZ (n=14)	STZ-Injected Rat					
			High Dose (60 mg/kg)			Low Dose (40 mg/kg)		
			STZ+I (n=15)	STZ (n=15)	Control (n=16)	STZ+I (n=13)	STZ (n=13)	Control (n=13)
Weight, g								
Initial	373±9	325±25	347±26	333±14	322±3	348±28	332±14	318±6
At death	452±13	363±14	387±31	267±18	382±10	387±32	297±14	379±11
Glucose, mg%	229±21*	173±36	251±41*	428±66*	174±26	221±18*	303±18*	162±19
Triglycerides, mg%	549±241*	71±16	60±23	852±624*	70±15	60±24	134±18*	70±14
Cholesterol, mg%	84±17	63±10	85±21	161±45*	84±7	82±21	103±9*	83±6
Insulin, ng/mL	3.74±3.16*	1.06±0.38	5.99±3.38*	0.58±0.18	1.05±0.42	4.32±1.62*	0.87±0.30*	1.39±0.45
Ketone (+)	0	0	0	13*	0	0	2	0

OZ indicates obese Zucker rat; LZ, lean Zucker rat; STZ+I, streptozotocin-injected rats with insulin treatment; and STZ, streptozotocin-injected rats without insulin treatment.

* $P<0.01$ vs control group in each diabetic model.

number of BrdU-labeled cells and the BrdU-labeling index was highest in obese Zucker rats relative to control lean Zucker rats at 7 days (202.27 ± 98.86 versus 35.71 ± 20.54 cells per cross section, $P<0.05$; $77.8 \pm 6.89\%$ versus $40.63 \pm 6.75\%$ index, $P<0.01$) and significantly higher in obese Zucker rats at 14 days (111.80 ± 30.44 versus 54.45 ± 20.22 cells per cross section, $P<0.01$; $47.81 \pm 8.80\%$ versus $38.80 \pm 7.82\%$ index, $P<0.05$) (Figure 3). By 21 days after injury, cell proliferation in the intima of obese Zucker rats had diminished to the level of lean Zucker rats. In both obese and lean Zucker rats, cell proliferation peaked in the media at 3 days (118.66 ± 84.28 and 27.50 ± 12.75 BrdU-labeled cells per cross section). In the intima, cell proliferation was markedly increased at 3 days and persisted through days 7 and 14 in both the obese and lean Zucker rats (202.27 ± 98.86 and 35.71 ± 20.54 BrdU-labeled cells per cross section at 7 days).

Discussion

Restenosis remains a major limitation of percutaneous coronary revascularization for patients with diabetes, and the factors contributing to this risk remain poorly defined. In this study, we assessed both type I and type II rat models for diabetes. There was not an increased propensity to form neointima in the type I diabetic rat model compared with

controls. The Zucker rat model, which is a prototype for type II diabetes, however, exhibited a marked tendency to form neointima after balloon injury compared with the lean controls. In both models, the rats had increased levels of serum glucose, cholesterol, and triglycerides, which were attenuated with exogenous insulin administration in the type I model compared with control rats. There were also increased circulating insulin levels in the type II and insulin-treated type I models compared with respective controls.

Diabetes and Restenosis

Patients with a reported history of diabetes mellitus account for 15% to 25% of those undergoing percutaneous coronary intervention procedures. Well over 90% of these patients have type II diabetes.

Unfortunately, there are currently no adequately characterized animal models to investigate the vascular response to injury in diabetes.

Generally, restenosis after balloon injury is a function of early elastic recoil, adverse remodeling, and the formation of neointima. The advent of stenting has favorably affected the rate of restenosis among diabetic and nondiabetic patients, primarily by reducing elastic recoil and adverse remodeling. Even in the present stent era, diabetes is associated with an increased risk of restenosis. This is a direct result of the

TABLE 2. Morphometry 21 Days After Carotid Balloon Injury in Each Diabetic Rat Model

	OZ (n=12)	LZ (n=14)	STZ-Injected Rat					
			High Dose (60 mg/kg)			Low Dose (40 mg/kg)		
			STZ+I (n=15)	STZ (n=15)	Control (n=16)	STZ+I (n=13)	STZ (n=13)	Control (n=13)
Intima, mm ²	0.21±0.06*	0.08±0.03	0.09±0.04	0.09±0.04	0.11±0.03	0.10±0.04	0.08±0.04	0.10±0.05
Media, mm ²	0.13±0.03	0.12±0.02	0.12±0.02	0.12±0.03	0.13±0.02	0.13±0.02	0.12±0.02	0.13±0.02
Lumen, mm ²	0.23±0.05	0.28±0.09	0.34±0.11	0.33±0.12	0.33±0.11	0.34±0.10	0.34±0.10	0.32±0.11
External elastic lamina, mm ²	0.58±0.01†	0.48±0.01	0.52±0.09	0.55±0.16	0.57±0.13	0.56±0.10	0.54±0.13	0.56±0.14

Abbreviations as in Table 1.

* $P<0.01$, † $P<0.05$ vs control group in each diabetic model.

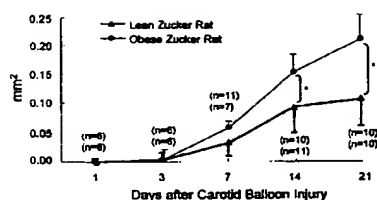


Figure 1. Changes of neointimal area after carotid balloon injury. Neointimal area progressively increased in type II diabetic obese Zucker rats vs lean Zucker rats beginning 7 days after injury. * $P<0.01$.

formation of neointima; thus, it has become imperative to develop, characterize, and ultimately modulate a diabetic model that demonstrates a propensity to form neointima. In the diabetic state, several mechanisms, such as enhanced coagulability, platelet hypersensitivity, dyslipidemia, or dysregulation of chemotactic factor expression, might combine to produce enhanced intimal hyperplasia.¹² All these putative mechanisms may be influenced by hyperglycemia, hyperinsulinemia, or insulin resistance.

Previous Animal Studies

There have been conflicting results in previous diabetic animal studies regarding the association of increased neointimal proliferation after arterial injury and the different types of diabetes. Two previous studies in type I diabetic models (Alloxan-induced diabetic rabbit¹³ and BB Wistar diabetic rat¹⁴) demonstrated increased intimal thickening after balloon injury compared with nondiabetic animals. In contradistinction, increased aortic intimal thickening was observed in the obese Zucker type II diabetic rats compared with the low-dose STZ-treated, insulin-treated Wistar diabetic rats.¹⁵ Our data also found an increase in neointimal proliferation after balloon injury in the obese Zucker rats. To characterize this model more completely, we included both insulin-treated rat and insulin-untreated rat groups in our study. There was no association, however, between neointimal hyperplasia and exogenous insulin administration, insulin deficiency, glucose, lipid, or triglyceride levels. Our study suggests an association of intimal hyperplasia and insulin resistance. In short, the underpinnings of proliferation after injury remain undetermined; however, further analysis with an insulin-resistant state seems warranted.

Diabetes results in increased circulating serum glucose levels. Enhanced glucose control has been shown to reduce microvascular complications in both type I¹⁶ and type II patients.¹⁷ Improvement in macrovascular complications, however, was not demonstrated in these trials. Hyperglyce-

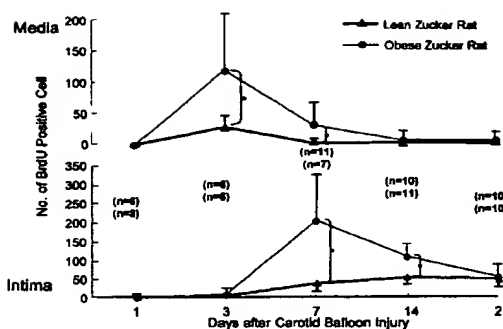


Figure 3. Number of BrdU-positive cells located in intima and media after balloon injury in Zucker obese and lean rats. * $P<0.05$.

mia might potentiate the response to arterial injury and may affect the initial step of restenosis.²¹ There is a significant correlation between thromboxane A₂ production, fasting plasma glucose, or hemoglobin A_{1c}¹⁸ levels and the expression of several growth factors.¹⁹ Hyperglycemia induces an increase in selected matrix gene transcriptions that persists for weeks after restoration of normoglycemia in vivo.²⁰ Furthermore, advanced glycosylation end products can mediate inflammatory cell recruitment and activation, stimulation of smooth muscle cell proliferation, and abnormal matrix production, all of which may promote restenosis. The degree of early nonenzymatic glycosylation is determined mainly by the glucose concentration.²¹ Formation of advanced glycosylation end products results from prolonged hyperglycemia and enhanced oxidative state. Inhibition of advanced glycosylation end products has been shown to decrease de novo atherosclerotic formation in the STZ-induced apolipoprotein E-null mouse model.²² Although hyperglycemia is potentially related to many steps in the process of restenosis, at present, no consistent data implicate hyperglycemia and restenosis.

Insulin has several biological actions, which may be related to the process of restenosis. Insulin exerts adverse effects on the balance between thrombosis and fibrinolysis by modulating the plasminogen activator and inhibitor systems.²³ Insulin can potentiate proliferation and migration of smooth muscle cells, most likely through the action of insulin-like growth factor²⁴ or other stimulatory factors.²⁵ Insulin also aggravates diabetic dyslipidemia²⁶ and theoretically promotes long-term recoil of the overstretched arteries.¹⁵ Although insulin and insulin-like growth factors are mitogenic, controversy remains as to the importance of insulin in promoting smooth

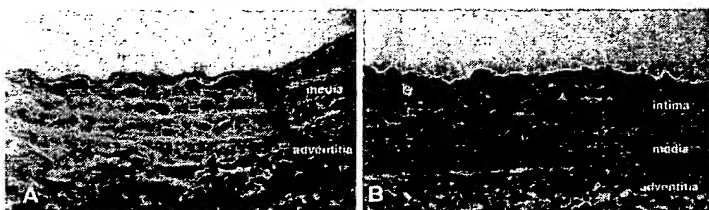


Figure 2. Photomicrograph of proliferative cells in media and intima after carotid balloon injury. Number of BrdU-labeled cells was significantly higher in the obese Zucker rat (B) than the lean Zucker rat (A) after balloon injury.

muscle cell proliferation and whether insulin alone or insulin resistance syndrome is key in modulating restenosis.

Insulin resistance is probably only a modest predictor of macrovascular disease,²⁷ and whether it is associated with increased restenosis is uncertain. Clinically, insulin resistance is characterized by the presence of diabetes, hypertension, obesity, and dyslipidemia. There are also suggestive data in humans that insulin-resistance syndrome is associated with restenosis among nondiabetic patients.^{28,29} Among nondiabetic insulin-resistant patients, Nishimoto et al²⁸ determined that insulin resistance was associated with an increased risk for restenosis after angioplasty. Furthermore, in EPISTENT,³⁰ patients with insulin resistance syndrome (defined as diabetes mellitus, hypertension, and obesity) had a higher rate of 6-month target vessel revascularization than the non-insulin-resistant cohort (16.7% versus 7.5%, $P < 0.001$). Likewise, data for this project would suggest that it is the insulin-resistant state rather than diabetes that results in the formation of neointima.

Study Limitations

This study has several limitations. The relevance of restenotic animal models to human restenosis is unknown, and no single model has yet been shown to reliably predict restenosis in humans. In fact, it is unlikely that any one model will be entirely explanatory of the human response to injury. Animal studies, however, are likely to provide important insights into the pathophysiology of vascular injury.

Conclusions

The obese Zucker rat carotid artery injury model appears to be an appropriate animal model for the study of the mechanism and treatment of increased restenosis associated with type II diabetes. The data from this study would suggest that an insulin-resistant model is associated with a propensity for neointimal proliferation. This was not seen in an insulin-deficient model with or without exogenous insulin administration.

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The effects of uncontrolled hyperglycemia on thrombosis and formation of neointima after coronary stent placement in a novel diabetic porcine model of restenosis

Andrew J. Carter^a, Lynn Bailey^a, Jennifer Devries^a and Brad Hubbard^b

Background Results of recent clinical studies suggest that patients with diabetes mellitus have a higher than normal rate of restenosis after percutaneous transluminal coronary angioplasty or coronary stenting. The mechanism for this exaggerated neointimal response is not known.

Objectives To determine the technical feasibility of a model of in-stent restenosis in swine with streptozotocin-induced hyperglycemia and to compare the late arterial responses to injury induced by placement of oversized coronary stents in diabetic and nondiabetic animals.

Methods Eighteen 25–40 kg castrated male or intact female Yucatan miniature swine aged 6 months were obtained from a commercial supplier. Twelve of the miniature swine were randomly selected for intravenous treatment with 125 mg/kg streptozotocin to induce a hyperglycemic state. Twelve weeks after treatment, all animals underwent placement of oversized balloon-expandable stainless steel stents in the coronary arteries. After 28 days, histomorphometric analysis of the stented coronary arteries to determine the neointimal responses for the diabetic and nondiabetic animals was completed.

Results Sudden death due to stent thrombosis occurred for five of 11 (45%) of the diabetic animals and none of the age-matched nondiabetic control animals ($P=0.05$). For histology after 28 days, the neointimal response was correlated to the extent of arterial injury for the diabetic ($r=0.79$, $P=0.0001$) and nondiabetic ($r=0.86$, $P=0.0001$) animals. The surviving diabetic animals had areas of neointimal ($1.67 \pm 0.74 \text{ mm}^2$) and percentages of in-stent stenosis (28 ± 14) similar to those of the nondiabetic swine ($1.36 \pm 0.40 \text{ mm}^2$, $P=0.26$; 22 ± 6 , $P=0.17$). Multiple regression analysis also demonstrated that arterial injury ($P=0.0001$) alone, not hyperglycemia ($P=0.237$), was independently correlated to formation of neointima.

Conclusions Uncontrolled hyperglycemia results in greater than normal thrombosis after coronary-stent placement in swine with streptozotocin-induced diabetes. These data suggest that greater than normal early formation of thrombus rather than proliferation of smooth muscle cells contributes to restenosis after coronary stenting in patients with diabetes mellitus. *Coron Artery Dis* 11:473–479 © 2000 Lippincott Williams & Wilkins.

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Keywords: stents, restenosis, diabetes

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Introduction

Results of recent clinical studies suggest that patients with diabetes mellitus have a higher than normal rate of restenosis after percutaneous transluminal coronary angioplasty or coronary stenting 1–3. Restenosis after percutaneous transluminal coronary angioplasty or stenting results from a combination of mechanical forces, formation of thrombus, inflammation, proliferation of cells, and late vascular remodeling 4–6. Coronary stenting eliminates immediate recoil of vessels and late constrictive remodeling 7,8. Therefore, formation of neointima is the principal cause of in-stent restenosis. Results of clinical intravascular ultrasound studies suggest that diabetic patients have an exaggerated neointimal response after coronary-stent placement, resulting in a greater than normal incidence of in-stent restenosis 9. The mechanism for this exaggerated neointimal response is not known.

Experimental models of restenosis in pigs have been useful in helping to determine the mechanism of restenosis after balloon angioplasty and stenting 10–15. Therefore, the development of an animal model of in-stent restenosis in diabetic swine might allow characterization of the local vascular response to injury, such as formation of a mural thrombus, inflammation, proliferation of cells, and expression of growth factors, which contribute to the neointimal response to arterial injury in an uncontrolled hyperglycemic state. Such data are critical in order to delineate the cellular events leading to restenosis of stents in patients with diabetes.

The objectives of this study were to determine the technical feasibility of a model of in-stent restenosis in swine with streptozotocin-induced hyperglycemia and to compare the late arterial responses to injury induced

by placement of oversized coronary stents in diabetic and nondiabetic animals. It was hypothesized that an uncontrolled hyperglycemic state exaggerates formation of neointima in response to placement of oversized coronary stents in a porcine model of in-stent restenosis.

Methods

Experimental design and general procedures

Animal research was initiated after approval by the Institutional Animal Care and Use Committee (IACUC) and conformed to the guidelines on animal research of the American Heart Association. Eighteen 25–40 kg castrated male or female Yucatan miniature swine aged 6 months were obtained from a commercial supplier (Lone Star Farms, Seguin, Texas, USA). Twelve of the miniature swine were randomly selected for treatment with 125 mg/kg streptozotocin (Zanosar; Pharmacia Upjohn, Kalamazoo, Michigan, USA), administered intravenously via a marginal ear vein to induce a hyperglycemic state [16,17]. All animals were then fed a normal diet for 12 weeks prior to undergoing coronary-stent placement. Fasting blood samples were obtained to measure complete blood counts (Coulter Gen S, Fullerton, California, USA) and levels of glucose, electrolytes, blood urea nitrogen, creatinine, total cholesterol, low-density lipoprotein cholesterol, triglycerides, total protein, and albumin using an automated analysis system (Hitachi 911 or 917, Hitachi reagents; Roche Diagnostics Co., Basel, Switzerland).

Stent placement

Animals were medicated orally with 650 mg aspirin and 30 mg extended-release nifedipine the evening prior to stent placement. An 8 F sheath was placed retrogradely in the right carotid artery, and 150 U/kg heparin was administered intra-arterially to animals under general anesthesia in order to achieve an activated clotting time greater than 300 s (Hemochron; International Technology, Edison, New Jersey, USA). Forty-two stainless-steel balloon-expandable stents (MULTI-LINK, Guidant, Inc., Santa Clara, California, USA) were implanted in the proximal or middle left anterior descending, left circumflex, and right coronary arteries using the guiding catheter as a reference in order to obtain a 1:1.1–1.2 stent:artery ratio compared with the baseline vessel diameter. Each animal received one, two, or three coronary stents. All of the stents were mounted on a 3.0–4.0 mm diameter compliant balloon for deployment. Placement of the stents was completed with a single balloon inflation at 810 kPa for 30 s. Angiography was completed after implantation to confirm patency of the stent. Animals were allowed to recover and were returned to care facilities, where they were fed a normal diet and 81 mg aspirin daily. The animals were returned

to the research catheterization laboratory for follow-up coronary angiography 28 days after implantation. After completion of angiography the animals were killed with a lethal dose of barbiturate.

Pathologic evaluation

Immediately after animals had been killed, the hearts were harvested, and the coronary arteries were perfusion fixed with 10% neutral buffered formalin at 60–80 mmHg for 30 min via the aortic stump. The stented segments of coronary artery were carefully dissected from the epicardial surface and embedded in methyl-methacrylate for sectioning. From each stent proximal, middle, and distal segments 1 mm thick were cut by a diamond-edged rotating saw. The stented segments were cut with a stainless-steel carbide knife at 4–5 μ m thickness. All histologic sections were stained with hematoxylin–eosin and elastic van Gieson stains. The cross-sectional areas of proximal, middle, and distal stent sections were measured with computerized digital morphometry (Optimas 6.1; Bothel, Washington, USA) to determine the areas within the external elastic lamina (EEL), internal elastic lamina (IEL), stent, and the vessel lumen. The percentage area stenosis was then defined as (IEL or stent area–lumen area) (IEL or stent area) $\times 100$. Area of neointima was determined by subtracting the area of the lumen from the area within the stent wires or IEL. Thickness of neointima extending perpendicularly from above the stent to the lumen surface was measured at each wire site. The severity of stent-induced vascular injury at each wire site was graded using the method of Schwartz *et al.* [10]. Briefly, the degree of injury at each wire site was assessed as grade 0, IEL intact with media compressed; grade 1, IEL lacerated with media compressed; grade 2, IEL and media lacerated with the EEL intact; and grade 3, EEL lacerated. The mean injury score for each arterial segment was calculated by dividing the sum of injury scores for each wire site by the total number of wires from the stent sections.

The stent sections from the animals that unexpectedly died suddenly were carefully examined to identify stent thrombosis as the etiology. Stent thrombosis was defined as the presence of an occlusive mural thrombus within the stent or a section adjacent to the stent. In addition, all sections were evaluated to identify anatomical factors, such as severe medial dissection (> 25% medial laceration), grade 2 or 3 stent-induced injury, and subintimal or medial hematoma, which might contribute to stent thrombosis.

Statistical analysis

Categorical data were compared by χ^2 analysis. The mean morphologic data for each group were compared

by analysis of variance with post-hoc analysis for multiple comparisons. Linear regression analysis was applied to examine the correlations between the morphometric variables. Multiple regression analysis was applied to assess the impact of arterial injury and diabetes, and their interaction term, on area of neointima. $P = 0.05$ was considered statistically significant. Data are expressed as means \pm SD. All statistics were calculated using Statview 4.5 (Abacus, Berkeley, California, USA) or the SAS statistical package (SAS Institute, Cary, North Carolina, USA).

Results

General

Of the 12 animals treated with streptozotocin 11 (92%) survived to undergo coronary-stent placement. One animal died 2 weeks after streptozotocin therapy due to acute renal failure. All of the remaining animals thrived during the 12-week interval preceding stent implantation. The mean total body weights for the diabetic (34.4 \pm 4.3 kg) and nondiabetic (32.0 \pm 3.1 kg, $P = 0.36$) animals were similar. The fasting serum lipid profiles for the diabetic and nondiabetic animals are summarized in Table 1. The mean fasting level of blood glucose was significantly greater for the streptozotocin-treated diabetic (362 \pm 93 mg/dl) than it was for the normal (76 \pm 6 mg/dl, $P = 0.0001$) swine. All of the diabetic (mean level of serum creatinine 0.74 \pm 0.11 mg/dl) and nondiabetic (mean level of serum creatinine 0.53 \pm 0.08 mg/dl) swine had normal renal function.

Procedural

All of the 42 MULTI-LINK stents (100%) were successfully implanted in the left anterior descending ($n = 16$, 38%), left circumflex ($n = 11$, 26%), or right ($n = 15$, 36%) coronary arteries of 11 diabetic ($n = 27$, 64%) and six nondiabetic ($n = 15$, 36%) swine. Five (45%) of the 11 diabetic animals and none of the nondiabetic animals died suddenly after stent implantation ($P = 0.05$). The sudden deaths of animals occurred 24–96 h after operation, after we had observed their normal recovery from anesthesia in all cases. Postmortem examination demonstrated that stent thrombosis was the cause of sudden

death for each of the diabetic animals that died. The metabolic parameters for the diabetic animals that died suddenly were similar to those of the surviving diabetic pigs.

Histology

Sudden death

Thirty-nine sections from 13 stented coronary arteries in the five diabetic animals that died suddenly were analyzed. An occlusive platelet-fibrin-rich thrombus was identified in at least one stented coronary artery (left anterior descending $n = 4$, left circumflex $n = 1$, right coronary $n = 1$) from each of the animals that died suddenly (Fig. 1). Histologic analysis confirmed that stent thrombosis had occurred in six (46%) of the 13 stented segments of coronary artery from the animals that died suddenly. Thus, stent thrombosis occurred in six (22%) of 27 stents implanted in the diabetic animals and in none (0%, 28-day-patency data) of the 15 stents implanted in the nondiabetic control animals ($P = 0.048$).

All of the stent sections examined that originated from animals that died suddenly were carefully evaluated to identify anatomical factors that may have contributed to stent thrombosis. In all 39 sections from within the 13 stents examined, the struts were apposed to the vessel wall with focal medial compression by the stent wire. Severe arterial injury or medial laceration (grade 2 injury) was present in only one of the 39 stent sections analyzed. For this stent there was evidence of occlusive thrombosis. A focal medial dissection (>25% of media) was identified in a section proximal or distal to the stent in two of the six arteries with occlusive thrombus and one of the seven arteries with patent stents ($P = 0.56$). There were no cases of severe medial dissection (>25% of media) or subintimal hematoma proximal or distal to the stents.

Neointimal response in diabetic versus non-diabetic swine

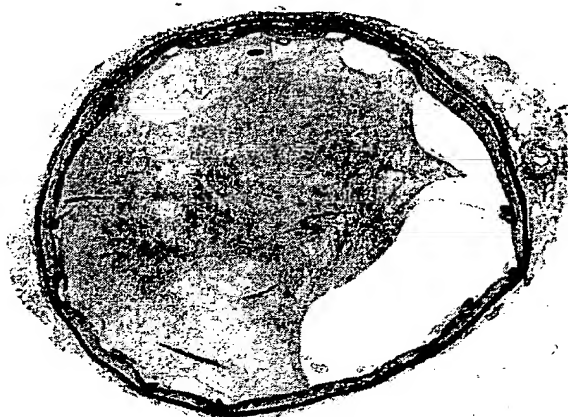
The morphometric data from the vessels analyzed 28 days after coronary-stent placement are summarized in Table 2. Three of the 29 vessels had severe stent-

Table 1 Summary of fasting serum lipid profiles for 11 diabetic and six nondiabetic Yucatan miniature swine

Group	Total cholesterol (mg/dl)	Low-density cholesterol (mg/dl)	High density cholesterol (mg/dl)	Triglycerides (mg/dl)
Diabetic ($n = 11$)	88 \pm 14**	30 \pm 15	44 \pm 7	77 \pm 42**
Normal ($n = 6$)	64 \pm 12	21 \pm 8	34 \pm 3	44 \pm 14

Data are expressed as mean \pm SD. ** $P = 0.003$ versus normal.

Fig. 1



Low-power photomicrographs of a stented coronary artery from a diabetic animal that died suddenly after stent placement. A large platelet-fibrin-rich thrombus obstructs the stent lumen (elastic van Gieson stain; $\times 10$ magnification).

induced arterial injury (grade 3, rupture of the EEL) and were omitted from the morphometric analysis. The cellular appearance of the neointima was similar for the diabetic and nondiabetic animals (Fig. 2). In general, the neointima consisted of well-organized smooth muscle cells and matrix proteins. Occasional regions of remnant fibrin thrombus were identified adjacent to the stent struts. The degree of inflammation was mild and similar for the diabetic and control animals (data not shown). The adventitia of the stented arteries for the diabetic and nondiabetic animals were also similar, with neovascular channels contained in a dense circumferential area of connective tissue.

The mean injury scores for the surviving diabetic (0.3 \pm 0.2) and nondiabetic (0.3 \pm 0.2, $P = 0.51$) animals were similar. Thicknesses of neointima at each wire site for the diabetic (186 \pm 110 μm) and nondiabetic (149 \pm 60 μm , $P = 0.08$) pigs were similar. The diabetic animals had similar areas of neointima and degrees of in-stent stenosis to the nondiabetic animals (Table 2). Linear regression modeling demonstrated that there was a strong correlation between area of neointima and arte-

rial injury for the diabetic ($r = 0.79$, $P = 0.0001$) and nondiabetic ($r = 0.86$, $P = 0.0001$) animals (Fig. 3). The plot of area of neointima versus injury illustrates neointimal responses to injury of the diabetic and nondiabetic pigs. Multiple regression analysis indicates that arterial injury alone ($P = 0.0001$), not hyperglycemia ($P = 0.24$) and not the interaction term (hyperglycemia \times injury, $P = 0.41$), was correlated to formation of neointima.

Discussion

In the present study we describe a novel experimental model of in-stent restenosis in diabetic swine, which allows characterization of the vascular reparative events after coronary stenting in a hyperglycemic state. Streptozotocin induces a unique hyperglycemic hypoinsulinemic state in animals that is consistent with type-I diabetes [16,17]. This allows evaluation of the effects of hyperglycemia on thrombosis and formation of neointima after coronary stenting in the absence of spontaneous or therapeutic hyperinsulinemic conditions. In the present study, the hyperglycemic animals had a substantially greater incidence of stent thrombosis than did age-matched nondiabetic swine. The metabolic status of the animal, however, was not an independent predictor of late formation of neointima. After 28 days, the neointimal response was correlated to the extent of stent-induced arterial injury but not the presence of hyperglycemia for the surviving diabetic and nondiabetic swine. These experimental data suggest that thrombus plays a critical role after coronary-stent placement with uncontrolled hyperglycemia and this could be useful for understanding clinical observations of stenting in diabetic patients who have a greater than normal risk of stent thrombosis and restenosis [1,3–5,9,18].

Stent thrombosis

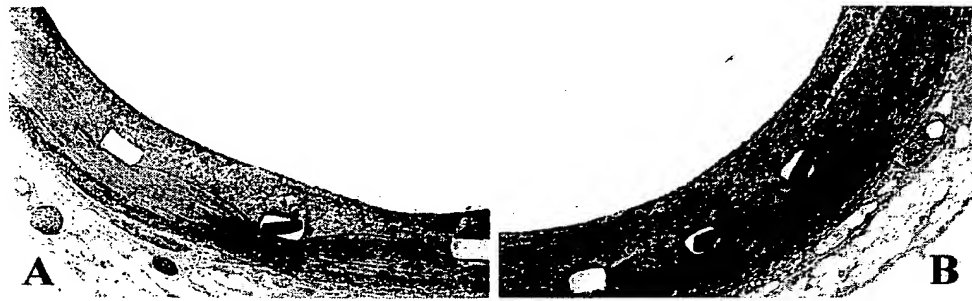
Diabetes is associated with several hematologic and vascular derangements that increase the risk of arterial thrombosis after percutaneous coronary interventions [18,19]. Hyperglycemia alters viscosity of blood, levels of clotting factors, aggregability of platelets and en-

Table 2 Summary of vessel morphometry 28 days after placement of stents in 26 coronary arteries of diabetic and nondiabetic minipigs

Group	Area of IEL or stent (mm^2)		Area of neointima (mm^2)		Area of lumen (mm^2)		Area stenosis (%)		Injury score	
Diabetic ($n = 14$)	6.13	1.03	1.67	0.74	4.46	1.28	28	14	0.3	0.2
Normal ($n = 12$)	6.33	0.79	1.36	0.40	4.97	0.77	22	6	0.3	0.2

Data are expressed as means \pm SD. IEL, internal elastic lamina.

Fig. 2



High-power photomicrographs 28 days after placement of stents in the coronary arteries of age-matched nondiabetic control animals (A) and animals with streptozotocin-induced diabetes (B). The cellular appearances of the neointima and adventitia for the diabetic and nondiabetic animals are similar (elastic van Gieson stain; $\times 50$ magnification).

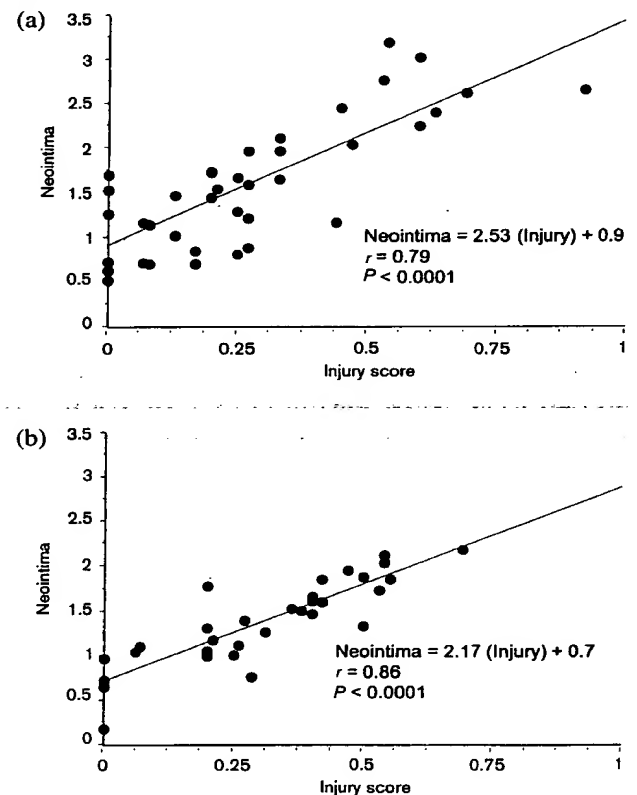
dothelial function, creating a procoagulant state 3,19–22. Elezi *et al.* 18 recently reported finding a significantly greater than normal incidence of major adverse cardiac events and subacute thrombosis during the first 30 days after coronary stenting in diabetic patients. Similar hematologic abnormalities have also been identified in diabetic animal models of atherosclerosis and vascular repair 20–23. In diabetic rats, accumulation of platelets is prolonged after endothelial denudation, resulting in a thicker neointima than that in nondiabetic rats 21. Colen *et al.* 22 reported a significantly worse than normal microvascular anastomotic thrombosis in diabetic rats that improved with glycemic control.

In the present study, the incidence of stent thrombosis was significantly greater for the streptozotocin-induced diabetic than it was for age-matched nondiabetic controls. The numbers of stents implanted per animal and procedural anticoagulation parameters for these two groups were similar. Furthermore, we could identify no anatomic factor, such as medial dissection or stent-induced arterial injury, to account for the greater incidence of sudden death due to stent thrombosis among the diabetic animals. These data suggest that hyperglycemia is associated with a marked prothrombotic state that incites stent thrombosis in the porcine coronary model. The histologic appearance of the occluded stents in the diabetic animals was consistent with an organizing platelet-fibrin thrombus typical of the porcine model and human coronary stent thrombosis 24,25. Therefore, this porcine diabetic model of restenosis could prove useful for screening systemic and local intracoronary-catheter based or stent-based therapies to prevent thrombosis as well as to determine the mechanisms causing greater than normal stent thrombosis in diabetics.

Results of recent clinical studies have suggested that periprocedural treatment with abciximab (c7E3,

ReoPro, Eli Lilly, Indianapolis, Indiana, USA), a monoclonal antibody to the platelet glycoprotein receptor

Fig. 3



Linear regression plots demonstrate that there is a strong correlation between formation of neointima and injury to arteries for diabetic (a) and nondiabetic (b) pigs. Multiple regression analysis demonstrated that arterial injury alone, not hyperglycemia and not the interaction term (hyperglycemia \times injury), was correlated to formation of neointima in the animals surviving 28 days. The y axis represents area of neointima in mm^2 .

glycoprotein IIb IIIa, significantly reduces incidences of early thrombotic complications and late restenosis among diabetic patients after coronary stenting 26. These data together with the present experimental study suggest that thrombus plays a critical role in determining clinical outcomes for diabetic patients after coronary stenting. Studies to evaluate platelet function and efficacy of platelet antagonists in preventing stent thrombosis in this diabetic model are planned.

Neointimal response to coronary stenting in diabetic swine

Hyperglycemia is associated with hypersensitivity of platelets to agonists, greater than normal synthesis of thromboxane, alteration of matrix-protein production, and upregulation of expression of growth factors that induce proliferation of smooth muscle cells. Hyperinsulinemia is also a potent stimulus for proliferation of smooth muscle cells that might contribute to an exaggerated neointimal response in diabetics after stenting 19. Clinical data, although they are inconsistent, suggest that diabetic patients have a greater than normal risk of restenosis after coronary-stent placement 1,5,18,27-29. Results of intravascular ultrasound studies implicate an exaggerated neointimal response to stenting as the factor causing greater than normal incidence of in-stent restenosis in diabetic patients 9. However, some investigators, such as Van Belle *et al.* 28, failed to demonstrate that there is a greater incidence of restenosis after coronary stenting among diabetic than there is among nondiabetic patients. The differences among late clinical outcomes in these studies of coronary stenting in diabetic patients are likely related to differences in study design, demographics, lesion characteristics, and, perhaps, individual variations in levels of glucose and insulin.

In the present study, the 28-day neointimal responses to oversized stenting in the surviving diabetic and with age-matched nondiabetic control swine were similar (Figs 2 and 3). These data suggest that the glycemic status of an animal need not independently affect the late neointimal proliferative response to coronary-stent placement. Hyperglycemia, however, is clearly associated with a markedly greater than normal incidence of stent thrombosis in the porcine model. Pigs are very susceptible to ischemic and embolically mediated ventricular fibrillation. In theory, if the diabetic animals that died suddenly due to excessive formation of thrombus survived the 28-day study, then, one might expect substantially greater late formation of neointima 24. Thus, the present results suggest that mechanisms other than an increase in proliferation of smooth muscle cells, such as the formation of a large thrombus matrix, may

contribute to causing restenosis after coronary stenting in diabetics. In addition, other factors, such as the atherosclerotic substrate, inflammation, and perhaps the presence of hyperinsulinemia may also be important in determining the neointimal response to stenting in patients with diabetes mellitus.

Limitations

Several important limitations apply to the present study. The animal model involves injury of normal coronary arteries in pigs 12 weeks after treatment with streptozotocin to induce hyperglycemia by nonspecifically destroying pancreatic cells. The histologic analysis was limited to a single 28-day time point, which is typical for the porcine model but might be insufficient to identify late progression of formation of neointima in hyperglycemic animals. The duration of hyperglycemia also might have been insufficient to affect the late biologic response to coronary-stent placement. The degree of hyperglycemia created in this animal model is similar to that in a patient with type-I or type-II diabetes mellitus 16,17. These diabetic pigs, however, do not have hyperinsulinemia, which is typical for a patient with type II diabetes. Hyperinsulinemia, in addition to having effects on the glycemic metabolic state, might contribute significantly to the exaggerated neointimal response to coronary stenting in diabetic patients. The prevalence of type II or adult-onset diabetes is far greater among patients undergoing intracoronary stenting than is that of type-I diabetes 3,4,18,19. Furthermore, diabetic patients often have smaller coronary arteries and more extensive atherosclerosis that may also contribute to the inferiority of post-procedural and long-term clinical outcomes with stenting relative to those of nondiabetic patients. The animals were treated only with aspirin, not the combination of aspirin and ticlopidine used to prevent stent thrombosis. Thrombosis of the corrugated-ring geometry stent (MULTI-LINK) used in this experimental model is rare using only aspirin antiplatelet therapy 6,30. Studies to evaluate the effects of platelet antagonists such as ticlopidine on stent thrombosis in the model are planned.

Despite the limitations, this model of in-stent restenosis in diabetic swine provides a unique opportunity for the evaluation of vascular reparative events after coronary-stent placement in a metabolic state of uncontrolled hyperglycemia. Importantly, uncontrolled hyperglycemia incites thrombosis after coronary-stent placement in swine with streptozotocin-induced diabetes. These data suggest that an increase in early formation of thrombus rather than proliferation of smooth muscle cells contributes to causing restenosis after coronary stenting in patients with diabetes mellitus.

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